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TECHNICAL ARTICLE

Comparative mitochondrial and nuclear quantitative PCR of historical marine mammal tissue, bone, baleen, and tooth samples

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Abstract

The use of historical and ancient tissue samples for genetic analysis is increasing, with ever greater numbers of samples proving to contain sufficient mitochondrial and even nuclear DNA for multilocus analysis. DNA yield, however, remains highly variable and largely unpredictable based solely on sample morphology or age. Quantification of DNA from historical and degraded samples can greatly improve efficiency of screening DNA extracts prior to attempting sequencing or genotyping, but requires sequence-specific quantitative polymerase chain reaction (qPCR) based assays to detect such minute quantities of degraded DNA. We present two qPCR assays for marine mammal DNA quantification, and results from analysis of DNA extracted from preserved soft tissues, bone, baleen, and tooth from several cetacean species. These two assays have been shown to amplify DNA from 26 marine mammal species representing 12 families of pinnipeds and cetaceans. Our results indicate that different tissues retain different ratios of mitochondrial to nuclear DNA, and may be more or less suitable for analysis of nuclear loci. Specifically, historical bone and tooth samples average 60-fold higher ratio of mitochondrial DNA to nuclear DNA than preserved fresh soft tissue, and the ratio is almost 8000-fold higher in baleen.

Keywords: ancient DNA, cetacea, DNA quantification, quantitative PCR, real-time PCR

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Introduction

Quantification of mitochondrial and/or nuclear DNA has become a critical tool for a variety of genetic and genomic studies, including ancient DNA (Wandeler *et al*. 2003; Alonso *et al*. 2004; Malmström *et al*. 2005; Coolen *et al*. 2006), population genetics (Morin *et al*. 2001; Nsubuga *et al*. 2004; Roeder *et al*. 2004), sex identification (Morin *et al*. 2005), cellular physiology (D'ez-Sánchez *et al*. 2003; Marcuello *et al*. 2005), and forensics (Alonso *et al*. 2004; Smith & Morin 2005). The current method of choice is quantitative polymerase chain reaction (qPCR), using either dual-labelled probes in a 5′ exonuclease assay (Holland *et al*. 1991; Livak *et al*. 1995; Smith *et al*. 2002), or more recently, DNA binding fluorescent dyes such as Sybr Green (Schneeberger *et al*. 1995; Becker *et al*. 1996). Quantitative PCR takes advantage of the properties of PCR, namely exponential amplification of a DNA target from as little as one to a few starting copies (Heid *et al*. 1996; Raeymaekers 1999). The results of the amplification are detected by the increase in fluorescence as probes are digested or double-stranded DNA concentration increases in the PCR, thus allowing accurate quantification of one or more DNA targets in a single-step assay. The size of the target to be amplified can also be very small, so that quantification of degraded DNA in noninvasive, historical, or ancient samples can be assessed for their potential use as a source of genetic material (Morin *et al*. 2001, 2006; Smith *et al*. 2002; Wandeler *et al*. 2003; Alonso *et al*. 2004).

One of the limitations of any PCR-based assay is the need for sequence specificity. This results in the need for conserved DNA regions for primer and probe annealing, both within and between species. To design such primers and probes, homologous sequences representing the variability of the target organisms need to be assessed, generally by multiple alignments of sequences from representative individuals or species. This has been shown to be

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effective previously for a variety of mammalian species for a nuclear qPCR assay based on the c-myc proto-oncogene, but variation in both primer and probe-binding regions indicated that it would be difficult to apply a single assay across a very wide phylogenetic distribution of species (Smith *et al*. 2002). Within a taxonomic group, however, it should be possible to find highly conserved sequences for qPCR primer design, especially if probe-based methods aren't used, so that the assay efficiency is less affected by sequence variation in the binding sites (Smith *et al*. 2002).

We present two new qPCR assays designed from multiple alignments of a single-copy nuclear locus, BMI-1, and from the mitochondrial 12s ribosomal gene, for quantification of marine mammal (cetacean and pinniped) DNA. These assays have been characterized for their ability to quantify DNA extracts from large numbers of marine mammal species and tissue types, including historical and ancient tissues. A variety of historical tissue types have been evaluated for characteristics of mitochondrial DNA (mtDNA) and single-copy nuclear DNA preservation.

Materials and methods

Samples and assays

A minimum of two samples from each of 26 species of 13 families of cetaceans and three families of pinnipeds from the Southwest Fisheries Science Center (SWFSC) DNA archive were used (Table 1). The DNA was extracted from tissue using standard methods: lithium chloride (Gemmell & Akiyama 1996), sodium chloride protein precipitation (modified from Miller *et al*. 1988), silica-based DNeasy kits (QIAGEN), and standard phenol/chloroform extraction. We also performed hard tissue extractions as described in Morin *et al*. (2006) using a silica protocol modified from Höss & Pääbo (1993) and Hofreiter *et al*. (2004). Two assays were designed for quantification: Cet12s for mtDNA and BMI-1 for nuclear DNA. The mtDNA Cet12s assay was designed by aligning sequences in the 12s ribosomal region using 48 sequences obtained from 13 families of Cetacea plus pig, pangolin and human sequences. Primers were designed using the program PRIMER EXPRESS (Applied Biosystems) to produce a 129-bp fragment (Table 2) which would amplify DNA from all cetaceans but not those from humans. The primers were used to produce the PCR product that was then cloned for establishing a standard curve and also for the amplification of the 129-bp product for quantification. The nuclear DNA BMI-1 assay was designed by aligning two cetacean sequences with those of 25 other mammalian species including a human for exclusionary purposes. Two external primers were chosen to amplify a 221-bp product for creating the insert to clone for the standard curve. Two internal primers were designed to create a 51-bp product for the qPCR assay (Table 2). All of the primers were designed to anneal at 58 °C. Amplification of both the nuclear and mtDNA fragments for the standard PCR was performed using a 50 μL reaction with a final concentration of 1× Bioline PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1% Triton 100), 2.0 mM MgCl, 250 μM dNTPs, 0.15 μM forward and reverse primers, and 0.02 U/μL *Taq*. Thermocycling was performed using an ABI 2720 thermocycler and the following parameters for standard PCR assays: an initial denaturing at 95 °C for 2 min and 30 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension for 5 min at 72 °C.

Cloning

The standard curve was developed using *Tursiops truncatus* linearized clones from the PCR product of the external primers sets for both Cet12s and BMI-1 (Table 2). The PCR products for both assays were obtained using the described protocol and purified with the QIAquick PCR Purification kit (QIAGEN). The clean PCR products were ligated with pCR2.1-TOPO plasmid vector and then transformed into TOP10 One Shot cells (TOPO TA cloning kit; Invitrogen). The cells were cultured overnight at 37 °C. Ten clones were chosen, and the DNA was isolated using a Miniprep Ultraclean kit (Mo Bio Laboratories). Plasmid inserts were verified by sequencing.

Clones were linearized using 20 U of the restriction enzyme *Hin*dIII (Invitrogen), and 3 μL of 5 mg/mL linear acrylamide followed by addition of 10 μL of 3M sodium acetate and 250 μL of 100% ethanol to coprecipitate the DNA and remove any contaminants such as unincorporated nucleic acids. After linearization, a 260/280 absorbance was measured and the copy number was calculated based on the number of nucleotides for both BMI-1 (4121 bp) and Cet12s (4029 bp). Clones were diluted to a 1×10^7 copies/μL concentration; then a serial dilution was performed to a 1×10^1 copies/μL concentration. Duplicates of these serial dilutions were used in each qPCR to create a standard curve to quantify nuclear DNA and mtDNA, respectively.

Assay development and testing

Quantitative PCR was carried out using Sybr Green fluorescence from two different methods. Amplification of 2 μL of DNA was performed using Stratagene's Brilliant SYBR Green QPCR core reagent kit (Catalogue nos 600546, 929546, La Jolla, CA, USA) and ABI's Power SYBR Green PCR Master mix (Catalogue no. 4367659, Foster City, CA, USA) on Stratagene's MX3000p. When using the Stratagene kit, we added Q buffer from QIAGEN's Hotstar *Taq* to a 0.5X final concentration. The cycling program for

Table 1 Families, species, tissue sources, and sample ID numbers for preserved fresh tissue samples assayed

Family	Species	Tissue source	Sample ID
Balaenidae	*Balaena mysticetus*	Harvest	6980
Balaenidae	*Eubalaena japonica*	Biopsy	13192
Balaenidae	*Eubalaena japonica*	Biopsy	43860
Balaenopteridae	*Balaenoptera physalus*	Biopsy	4632
Balaenopteridae	*Balaenoptera physalus*	Stranding	10743
Balaenopteridae	*Balaenoptera acutorostrata*	Strand	23633
Balaenopteridae	*Balaenoptera acutorostrata*	Stranding	2313
Balaenopteridae	*Balaenoptera borealis*	Biopsy	30479
Balaenopteridae	*Balaenoptera borealis*	Biopsy	4002
Balaenopteridae	*Balaenoptera edeni*	Biopsy	30407
Balaenopteridae	*Balaenoptera edeni*	Stranding	26363
Balaenopteridae	*Balaenoptera musculus*	Sloughed skin	9353
Balaenopteridae	*Balaenoptera musculus*	Tagging	29838
Balaenopteridae	*Megaptera novaeangliae*	Biopsy	28500
Balaenopteridae	*Megaptera novaeangliae*	Strand	2813
Delphinidae	*Delphinus tropicalis*	Biopsy	33703
Delphinidae	*Delphinus delphis*	Biopsy	38263
Delphinidae	*Globicephala. macrorhynchus*	Fishery	1685
Delphinidae	*Globicephala. macrorhynchus*	Strand	2819
Delphinidae	*Lagenorhynchus obliquidens*	Gillnet fishery	4817
Delphinidae	*Lagenorhynchus obliquidens*	Gillnet fishery	8757
Delphinidae	*Lissodelphis borealis*	Biopsy	26303
Delphinidae	*Lissodelphis borealis*	Gillnet fishery	23163
Delphinidae	*Orcinus orca*	Biopsy	40919
Delphinidae	*Orcinus orca*	Biopsy	26566
Delphinidae	*Stenella attenuata*	Gillnet fishery	2084
Delphinidae	*Stenella attenuata*	Gillnet fishery	2085
Delphinidae	*Tursiops truncatus*	Stranding	4366
Delphinidae	*Tursiops truncatus*	Stranding	4375
Eschrictidae	*Eschrichtius robustus*	Stranding	13329
Eschrictidae	*Eschrichtius robustus*	Stranding	14205
Eschrictidae	*Eschrichtius robustus*	Stranding	23327
Kogidae	*Kogia breviceps*	Stranding	10117
Kogidae	*Kogia breviceps*	Stranding	10119
Monodontidae	*Delphinapterus leucas*	Biopsy	26683
Monodontidae	*Delphinapterus leucas*	Stranding	49106
Otariidae	*Zalophus californianus*	Stranding	12651
Otariidae	*Zalophus californianus*	Stranding	12652
Phocidae	*Phoca vitulina*	Ak-Anhsc-harvest	45434
Phocidae	*Phoca vitulina*	Ak-Anhsc-harvest	45435
Phocoenidae	*Phocoenoides dalli*	Biopsy	7960
Phocoenidae	*Phocoenoides dalli*	Gillnet hishery	1880
Physeteridae	*Physter macrocephalus*	Biopsy	14155
Physeteridae	*Physter macrocephalus*	Biopsy	15966
Pinniped	*Eumetopias jubatus*	Tagging	45000
Pinniped	*Eumetopias jubatus*	Tagging	45001
Ziphiidae	*Mesoplodon densirostris*	Stranding	8681
Ziphiidae	*Mesoplodon densirostris*	Stranding	9110
Ziphiidae	*Mesoplodon stejnegeri*	Stranding	25178
Ziphiidae	*Ziphius cavirostris*	Biopsy	30071
Ziphiidae	*Ziphius cavirostris*	Fishery	1120

the qPCR was performed with the following parameters: an initial denaturing at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and an extension at 72 °C for 30 s.

Both assays were validated using seven samples: *T. truncatus*, *Orcinus orca*, *Balaena mysticetus*, *Phoca vitulina* (Table 1), human DNA concentrated at 231 ng/μL, a 1:10 and 1:100 dilution and one no template control (NTC). The

Table 2 Primer names, sequences and uses for Cet12s and BMI-1 assays

Assay	Primer	Sequence 5′–3′	Purpose
Cet12s	Cet12s-F2Q	AACTCAAGGACTTGGCGGTG	mtDNA cloning and qPCR
	Cet12s-R2Q	CAATCCATAGGTTACACCTTGACCTA	mtDNA cloning and qPCR
BMI-1	BMI1_MMSTD_F	TGTGAACCTGTAGAAAACAAGTGCT	Nuclear DNA cloning
	BMI1_MMSTD_R	CCCGCTTTCAGGATTACAGATT	Nuclear DNA cloning
	BMI1.MM.F	TTTAGCCATTTTGATTCCTGTTTG	Nuclear DNA qPCR
	BMI1.MM.R	TTCGCGTAGCAACAGAGAAGTAA	Nuclear DNA qPCR

sample amplification curves were compared to the standard curve on the MX3000p. Following assay validation, two DNA samples from each of the 26 species representing 13 families were tested. Samples with poor performance were diluted and amplification was repeated based on results indicating that the PCRs were inhibited by too much DNA or other PCR inhibitors. Additional DNA samples from bone, tooth, dried tissue from museum skeletons, and baleen were also analysed, along with extraction negative controls.

Statistical analysis

DNA concentrations were not normally distributed, so they were log-transformed prior to statistical analysis. Concentrations from groups of samples were compared using two tailed *T*-tests ($\alpha = 0.05$). For comparison of the ABI vs. Stratagene kits, a paired *T*-test was performed using only samples which amplified successfully from undiluted DNA using both methods ($N = 19$ for Cet12s, $N = 18$ for BMI1).

Amplification plot slopes (APS) were calculated from the raw fluorescence data (termed dR) from each sample or standard curve plasmid dilution by calculating the slope of a regression for each set of seven consecutive data collection points during the PCR cycling, and selecting the slope from the set of points with the highest correlation coefficient (R^2). Slopes were only used in comparisons among sample groups if $R^2 > 0.997$. Sets of slopes from the standard curve dilutions and the samples were compared within assays using one-tailed *T*-tests with assumption of unequal variances among sample sets. For the tissue samples, only samples which amplified successfully from undiluted DNA in both assays were used.

Results

Both the Cet12s and the BMI1 qPCR assays worked on at least one sample from all 26 marine mammal species tested, and with both the Stratagene and ABI qPCR protocols, with the exception of sperm whale (*Physeter macrocephalus*), which only amplified with the Stratagene protocol. Human DNA was evaluated with both assays at two concentrations [231 ng/μL (~1617 copies/μL) and 2.31 ng/μL] using the Stratagene protocol, and both concentrations failed to amplify with either assay.

Comparison of the two protocols indicates that efficiency (Klein *et al*. 1999; Smith *et al*. 2002) changes slightly based on protocol selection. With the Stratagene protocol, efficiencies were 81% and 91% for Cet12s and BMI1, respectively. With the ABI master mix, efficiencies were 86% and 85%, respectively. Inferred DNA concentration using the two methods didn't differ significantly between methods for either assay ($P > 0.05$), though individual sample concentrations did vary. These assays were set up at different times, so variation could be due to use of different equipment or other laboratory variables. For both assays and protocols, the standard curves had an $R^2 > 0.99$. For all samples except the killer whale teeth, we present further data analyses based on only the ABI master mix results.

All plasmid dilutions used for the standard curves amplified consistently, indicating a linear amplification range of at least seven orders of magnitude, from 10 to 10^7 copies/μL. Analysis of tooth and bone samples with very low DNA concentrations indicates that as little as one copy of DNA in a PCR mix can be detected (Fig. 1). However, some DNA samples extracted from tissue failed to amplify, or amplified poorly when used undiluted, but amplified at normal efficiency when diluted 10- to 100-fold. This, in addition to higher-than-normal initial Sybr Green signals at the beginning of the qPCR cycles, indicates that high concentrations of DNA can inhibit the qPCR assay. For the Cet12s assay, samples that required dilution typically were assessed to have > 250 000 copies/μL (average = 55 303 564 copies/μL, $N = 18$), whereas samples that amplified well without dilution were typically less than 1 million copies/μL (average = 742 417 copies/μL, $N = 28$).

As expected, the preserved fresh tissues produced significantly more DNA than the historical samples. Among modern samples, extraction methods varied, so comparison among tissue sources is not very meaningful. However, historical tissue samples were extracted using the same method, and differed significantly in yield for mtDNA but not for nuclear DNA. The average Log(Cet12s copies/μL) was 4.7 ($N = 11$) for baleen, which was significantly greater ($P < 0.001$) than for bowhead bone (3.5, $N = 28$), and for

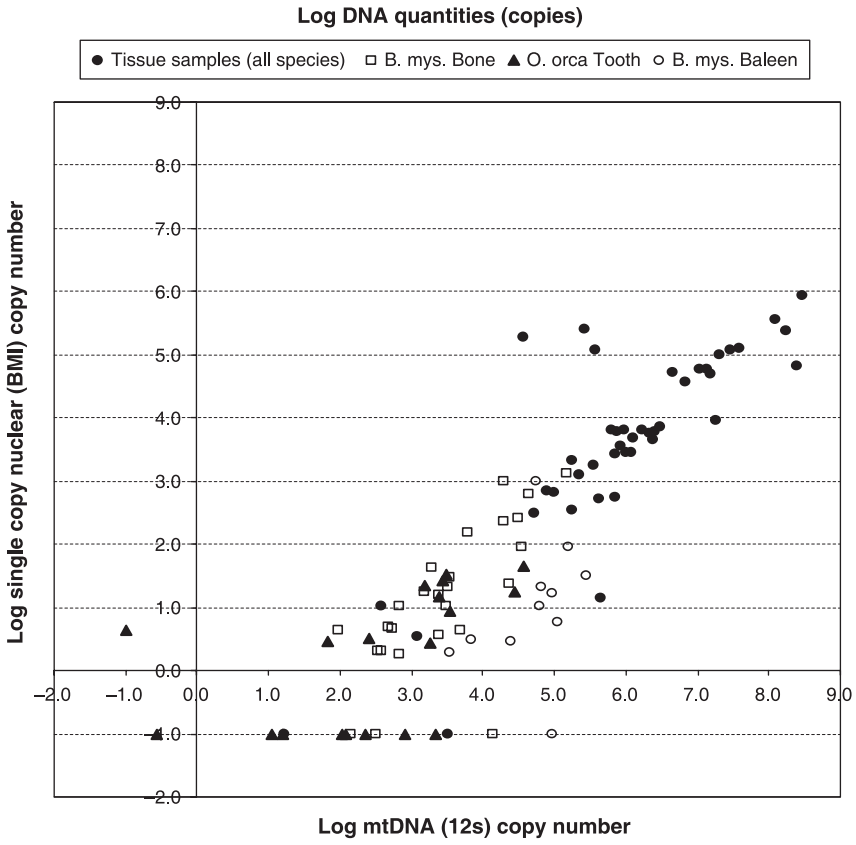

Fig. 1 Log of DNA copies/μL for the mtDNA (Cet12s) assay and the single-copy nuclear DNA (BMI-1) assay. As Log(0) is undefined, samples with no amplification in one of the assays were converted to a value of (–1) so that they could be plotted, while indicating that they did not have amplifiable DNA. The three samples with ratios of mtDNA to nuclear DNA less than 1 and high copy numbers had to be diluted 50–100 fold before amplification with the Cet12s assay.

Table 3 *P* values and samples sizes for comparisons of samples to standards within assays for each tissue type

	BMI *P* value	N (samples, standards)	Cet12S *P* value	N (samples, standards)
Preserved tissue	0.006	20, 12	0.004	23, 12
KW tooth & bone	0.003	24, 14	0.157	16, 12
Bowhead baleen and bone	0.111	21, 8	0.279	36, 12
Bowhead baleen only	0.114	6, 8	0.163	25, 12
Bowhead bone only	0.125	15, 8	0.033	11, 12

killer whale teeth (3.4, $N = 10$). Yields of nuclear DNA (Log(BMI-1 copies/μL) were 1.2, 1.4, and 1.1 for baleen, bone, and teeth, respectively. All extraction and PCR no-template controls showed no amplification with either assay.

Comparison of the ratio of mtDNA to nuclear DNA indicates that preserved fresh tissue has the lowest ratio (Log12s copies/μL/Log(BMI-1 copies/μL) = 1.8), and that this is significantly lower than the ratios for bone, baleen and teeth ($P < 0.01$). Bowhead bone and killer whale teeth yielded similar ratios (3.6 for both), and baleen had a marginally significantly higher ratio than bone or teeth at 5.7 ($P = 0.05$ and 0.1 for bone and teeth, respectively). With the killer whale samples, 13 of the 27 samples had positive amplification for the BMI assay resulting in inferred copy number < 10 copies/μL. Because amplification from one or a few copies of DNA can result in highly variable results (Raeymaekers 1999), these samples were excluded from comparisons of yields and ratios among sample sets.

We observed some indication of PCR inhibition from some samples of each type and for each assay. To determine whether assays were generally affected differentially by PCR inhibition, which could result in different apparent ratios of mtDNA to nuclear DNA, we compared the amplification plot slopes for differences in efficiency between the test samples and the standard curve dilutions (Smith *et al*. 2002) (Table 3). Standard plasmid dilutions showed no signs of PCR inhibition and were replicated at least twice for each dilution within each assay, and could thus be used as 'no inhibition controls' within assays. There was no indication of inhibition for either the BMI or the Cet12s assays for the Bowhead baleen samples, which had the highest

ratio of mtDNA to nuclear DNA. Bowhead bone samples, however, showed some inhibition of the Cet12s assay, which would tend to decrease the observed mtDNA to nuclear DNA ratio. For the killer whale tooth and bone samples, there was significant inhibition detected only for the BMI assay, and not for the 12s assay. This would tend to increase the observed ratio of mtDNA to single-copy nuclear DNA detected by these assays, indicating that the actual ratio for this sample type could be less than was observed. Surprisingly, the greatest inhibitory effects were detected in both of the assays when performed on DNA from preserved tissue samples. Both assays showed significant inhibition of samples relative to standard dilutions. However, because both assays appear to be similarly sensitive to PCR inhibition by something in these samples, there is no reason to believe the observed ratios of mtDNA to nuclear DNA are skewed.

Discussion

Accurate quantification of amplifiable DNA, especially when it is very dilute, can be critical for screening of samples prior to attempting to genotype or sequence, selection of appropriate samples (Morin *et al*. 2001, 2006; Wandeler *et al*. 2003), and diluting samples for consistent results. We have developed two qPCR assays which quantify mitochondrial and single-copy nuclear DNA from genomic DNA of marine mammals (cetaceans and pinnipeds). These assays will be beneficial in screening samples prior to attempting sequencing or genotyping, though the small fragment size assayed will be most relevant to similarly small fragments in highly degraded samples, such as for single nucleotide polymorphism (SNP) genotyping or sequencing of multiple overlapping small PCR products. Additionally, these assays do not quantify human DNA, so that potentially contaminating human DNA from handling of museum samples, for example, is not a problem (e.g. Wandeler *et al*. 2003).

Quantitative PCR assays typically work over a range of at least five to seven orders of magnitude. Our results indicate that this is true for these assays, but that sample characteristics may limit qPCR at higher concentrations. Given that the standard dilutions amplified successfully at 10^7 copies/μL, it is likely that either the greater complexity of genomic DNA vs. plasmid DNA or PCR inhibitors extracted with the genomic DNA are the cause of assay inhibition at higher genomic DNA concentrations. It should also be noted that efficiency differences among species has not been characterized in the current study. This could be done by analysis of amplification plot slopes (Smith *et al*. 2002) or dilution series of each species (Klein *et al*. 1999), but should not be necessary if absolute quantification is not the goal, but rather to select amplifiable DNA samples or compare among samples within a species.

One of the most interesting results of the current study was the observation of different ratios of mitochondrial to nuclear DNA among the different sample groups. Most samples had a ratio of approximately 300:1 in DNA from freshly preserved tissue samples. We would expect a ratio of approximately 1000:1 based on the number of copies of mtDNA found in human cells, though interspecies variability may be extensive, with as few as 10 copies per cell found in mice (D'ez-Sánchez *et al*. 2003; Timken *et al*. 2005). It is interesting that historical bone and tooth samples tended to have similar yields and ratios, but that baleen had a much higher ratio, indicating differential loss of nuclear DNA from this tissue type, either as the baleen is created or as it ages. Wandeler *et al*. (2003) showed that nuclear DNA degrades rapidly in tooth samples over approximately 5–30 years, with larger fragments (> 200 bp) disappearing within 10–20 years. Our data indicate that bone and tooth samples both have approximately 60-fold higher ratios of mtDNA to nuclear DNA than do preserved fresh tissues, indicating that, although these samples may retain both nuclear and mtDNA, the nuclear DNA may be degrading more quickly than mtDNA, or starts at lower quantities in these hard tissues. This pattern is even more extreme in baleen, with an almost 8000-fold higher ratio of mtDNA to nuclear DNA than was found in preserved fresh tissue. To our knowledge, no one has proposed a reason as to why these patterns may occur.

To test whether these observed differences were artefacts of qPCR inhibition, we analysed relative efficiency of amplification of samples compared to standard plasmid dilutions, and our results indicate that for baleen and preserved tissue samples, there is no reason to believe the observed ratios are affected by differential inhibition. For killer whale tooth and bone samples, it is possible that the elevated ratio (relative to preserved tissue) may be partly due to differential inhibition of the BMI assay, causing an artificially increased mtDNA to nuclear DNA ratio. On the other hand, bowhead bone showed the opposite effect, so it is difficult to know whether differential PCR inhibition is tissue-specific or some combination of other factors such as age, preservation conditions, tissue, etc. This should be investigated further both to determine the general patterns among tissue types and to determine the patterns of nuclear and mtDNA loss over time.

There remains the possibility that our mtDNA assay could be detecting both mtDNA and nuclear fragments (numts) of the mitochondrial 12s gene. Numts have been shown to be differentially amplified from some tissues (Greenwood & Pääbo 1999) and more common in some species than others (Thalmann *et al*. 2004), and this could cause an apparent increase in the ratio of mtDNA to nuclear DNA from those tissues or species. We are not aware of any published report that numts are common in cetaceans, or preferentially amplified from tooth, bone, or baleen.

Conserved primers, designed by means of aligned marine and terrestrial mammal sequences have been confirmed to work on all marine mammals tested to date, though one species (sperm whale) appears to work only under one of the two sets of conditions tested. These primers will allow rapid application of these methods to studies of noninvasive, historical, ancient, or otherwise poor quality or dilute DNA from any marine mammal, without the need to generate costly species-specific primers or probes. Finally, the use of Sybr Green for quantitative fluorescent detection serves to reduce the cost of assay reagents and simultaneously allows for the design of very small PCR products to detect highly degraded DNA, and reduced effects of sequence mismatch on the efficiency of amplification detection (Smith *et al*. 2002). Both qPCR assays proved to be useful tools for assessing the utility of marine mammal historical and ancient samples from various types of tissues for future genetic applications.

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References

Alonso A, Martín P, Albarrán C *et al.* (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Science International*, **139**, 141–149.

Becker A, Reith A, Napiwotzki J, Kadenbach B (1996) A quantitative method of determining initial amounts of DNA by polymerase chain reaction cycle titration using digital imaging and a novel DNA stain. *Analytical Biochemistry*, **237**, 204–207.

Coolen MJL, Boere A, Abbas B *et al.* (2006) Ancient DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediments from the Black Sea. *Paleoceanography* **21**, PA1005-doi: 1010.1029/2005PA001188.

Díez-Sánchez C, Ruiz-Pesini E, Lapeña AC *et al.* (2003) Mitochondrial DNA content of human spermatozoa. *Biology of Reproduction*, **68**, 180–185.

Gemmell NJ, Akiyama S (1996) An efficient method for the extraction of DNA from vertebrate tissues. *Trends in Genetics*, **12**, 338–339.

Greenwood AD, Pääbo S (1999) Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. *Molecular Ecology*, **8**, 133–137.

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real-time quantitative PCR. *Genomic Research*, **6**, 986–994.

Hofreiter M, Rabeder G, Jaenicke-Despres V *et al.* (2004) Evidence for reproductive isolation between cave bear populations. *Current Biology*, **14**, 40–43.

Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′-to-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences, USA*, **88**, 7276–7280.

Höss M, Pääbo S (1993) DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Research*, **21**, 3913–3914.

Klein D, Janda P, Steinborn R *et al.* (1999) Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification. *Electrophoresis*, **20**, 291–299.

Livak K, Flood SJA, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications*, **4**, 357–362.

Malmström H, Stora J, Dalén L, Holmlund G, Götherström A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. *Molecular Biology and Evolution*, **22**, 2040–2047.

Marcuello A, González-Alonso J, Calbet JA *et al.* (2005) Skeletal muscle mitochondrial DNA content in exercising humans. *Journal of Applied Physiology*, **99**, 1372–1377.

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, **16**, 1215.

Morin PA, Chambers KE, Boesch C, Vigilant L (2001) Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology*, **10**, 1835–1844.

Morin PA, LeDuc RG, Robertson KM *et al.* (2006) Genetic analysis of killer whale (*Orcinus orca*) historical bone and tooth samples to identify western U.S. ecotypes. *Marine Mammal Science*, **22**, 897–909.

Morin PA, Nestler A, Rubio-Cisneros NT, Robertson KM, Mesnick SL (2005) Interfamilial characterization of a region of the ZFX and ZFY genes facilitates sex determination in cetaceans and other mammals. *Molecular Ecology*, **14**, 3275–3286.

Nsubuga AM, Robbins MM, Roeder AD *et al.* (2004) Factors affecting the amount of genomic DNA extracted from ape feces and the identification of an improved sample storage method. *Molecular Ecology*, **13**, 2089–2094.

Raeymaekers L (1999) General principles of quantitative PCR. In: *Methods in Molecular Medicine* (eds Kochanowski B, Reischl U), pp. 31–41. Humana Press, Totowa, New Jersey.

Roeder AD, Archer FI, Poinar H, Morin PA (2004) A novel method

for collection and preservation of faeces for genetic studies. *Molecular Ecology Notes*, **4**, 761–764.

Schneeberger C, Speiser P, Kury F, Zeillinger R (1995) Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods and Applications*, **4**, 234–238.

Smith S, Morin PA (2005) Optimal storage conditions for highly dilute DNA samples: a role for trehalose as a preserving agent. *Journal of Forensic Sciences*, **50**, 1101–1108.

Smith S, Vigilant L, Morin PA (2002) The effects of sequence length and oligonucleotide mismatches on 5′ exonuclease assay efficiency. *Nucleic Acids Research*, **30**, e111.

Thalmann O, Hebler J, Poinar HN, Pääbo S, Vigilant L (2004) Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. *Molecular Ecology*, **13**, 321–335.

Timken MD, Swango KL, Orrego C, Buoncristiani MR (2005) A duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples: implications for quantifying DNA in degraded samples. *Journal of Forensic Science*, **50**, 1044–1060.

Wandeler P, Morin PA, Smith S, Pettifor RA, Funk SM (2003) Patterns of nuclear DNA degeneration over time — a case study in historic teeth samples. *Molecular Ecology*, **12**, 1087–1093.